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DNA Analysis of Seven Human Skeletons Excavated from the Terp of Wijnaldum

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DNA was extracted from the skeletal remains of seven individuals, six infants and one subadult, excavated from a terp near Wijnaldum, in Friesland (Netherlands). Their ages range from the 2nd to the 9th century AD and they were excavated under clean conditions to facilitate DNA analysis.

Mitochondrial DNA was amplified from the extracts and the first hypervariable segment of the control region was sequenced. A comparison between the terp inhabitants and modern Frisian speakers shows that the early medieval Wijnaldum inhabitants resemble modern islander Frisian speakers in having a high level of diversity, while the modern mainland Frisians are not distinguishable from the north-west German populations.

The amplification of a fragment of the Y chromosome allowed us to determine the genetic sex of the seven individuals. In the case of the subadult, our result is in agreement with the morphological data. For the six infants, DNA analysis is the only way to obtain this information.

Keywords: ANCIENT DNA, MITOCHONDRIAL DNA, FRISIANS, SEX DETERMINATION, NETWORKS.

Introduction

Terps are dwelling mounds found in the coastal area of the northern Netherlands and North Germany. The terp of Wijnaldum, situated in the Netherlands mainland, was inhabited from the 2nd or 3rd to the 9th century AD (Gerrets, 1995). Archaeological excavations in the early 1990s (Besteman, Bos & Heidinga, 1992, 1993) indicate that the terp was not an agricultural settlement but a centre of a rich élite. In the 5th century, there was a dramatic change in the architecture and material culture, with a predominance of Anglo-Saxon wares (Gerrets, 1995). This change appears in other terps such as Ezinge (van Giffen, 1936), but there is still controversy about whether it indicates an Anglo-Saxon migration or acculturation (Gerrets, 1995).

In 1992, the skeleton of a baby was found at Wijnaldum. In 1993, some other human remains were excavated, increasing the number of individuals to at least six newborns and one young adult (Pasveer, unpubl. data). The stratigraphical age estimates of these remains range from the 2nd to the 9th century AD (Cuijpers *et al.*, in press).

The polymerase chain reaction (PCR), has made possible the study of trace amounts of DNA which sometimes survive in archaeological remains (Mullis & Faloona, 1987; Hagelberg, Sykes & Hedges, 1989; Pääbo, Higuchi & Wilson, 1989). The study of ancient human remains is complicated by the numerous sources of contamination, caused by extensive manipulation of the remains. The modern DNA contaminant is likely to outcompete ancient degraded DNA during the PCR. In this study, the remains were newly excavated, and were directly sent to Oxford. Moreover, the excavation and subsequent manipulation of the bones was done with gloves, minimizing the risks of contamination.

Mitochondrial DNA (mtDNA) has been the preferred target for recovery from archaeological remains both because of its relative abundance in cells compared to nuclear DNA and because its unusual genetics lend themselves to population analysis (Wilson *et al.*, 1985). Ancient samples can be studied either by molecular typing (Hänni *et al.*, 1995) or sequencing. The sequence of human mtDNA has been determined (Anderson *et al.*, 1981) and its variation in modern European populations studied (Di Rienzo & Wilson, 1991; Piercy *et al.*, 1993; Pult *et al.*, 1994; Richards *et al.*, 1996). In particular, we have accumulated data from extant Frisian speakers from the North Frisian islands and the mainland of north-west Germany and the Netherlands (Richards *et al.*, 1995; Bandelt *et al.*,

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1995). In this context, an analysis of the mtDNA of the Wijnaldum remains would allow the introduction of a time dimension to these studies.

Sex determination is also possible using PCR. Currently two different methods are being used: the amplification of a Y chromosome repeat DYZ1 (Kogan, Doherty & Gitschier, 1987; Hummel & Hermann, 1991), or the amplification of part of the amelogenin gene, which has a different length in the Y and X chromosomes (Sullivan *et al.*, 1993; Faerman *et al.*, 1995). The sex of the subadult has been determined from morphological data (Cuijpers *et al.*, in press). In the case of the babies, DNA analysis is the only method by which the sex can be determined.

Materials and Methods

Materials

Six infant skeletons and one young adult were excavated using disposable gloves, so that they did not come into contact with living human tissue at any time. A fragment of humerus or femur of each individual and a tooth and bone lamella from the marrow cavity of the young adult were transferred from Groningen to Oxford in sterile plastic bags. The individuals are (Cuijpers *et al.*, in press): W93-11702, subadult of 18–19 years, diagnosed as female on basis of morphology, dated to the 6th century AD; W92-7379, very young infant, possibly stillborn, dated to the 7th or 8th century AD; W92-7946, comparable to 7379 or slightly older, dated to the 8th or 9th century AD; W92-8260, infant a few months old, dated to the 5th century AD; W93-9021, infant a few months old, dated to the 4th century AD; W92-STORT and W92-8008 come from scattered infant bones without association with other finds. It is likely that W92-8008 comes from the 2nd or 3rd century AD (D. Gerrets, pers. comm.)

Microscopy

Samples of bones were taken for histological examination in order to assess their state of preservation (Hedges, Millard & Pike, 1995; Richards, Sykes & Hedges, 1995). A fragment of each of the six infants, bones was embedded into epoxy resin, polished and examined at 100 × magnification.

Nitrogen

The N content was measured by mass spectrometry (Hedges, Millard & Pike, 1995), as an estimation of the collagen survival.

DNA extraction

The bone samples were cleaned and powdered in a coffee grinder, cleaned with sodium hypochlorite (Richards, Sykes & Hedges, 1995), but were not treated with sodium hypochlorite. The tooth was shotblasted and drilled using drill bits cleaned by soaking in 0·5% sodium hypochlorite solution for 15 min. Between 100 and 300 mg of powder (22 mg for the tooth) was incubated on a rotary shaker for 24 h at 50°C in 1 ml 0·5 M EDTA, pH 8, with Sarkosyl (BDH) to 0·5% and 1 unit of proteinase K (Sigma). Phage λ DNA was added as a carrier at a concentration of 1·35 µg per ml of EDTA. The DNA was then extracted with phenol, phenol/chloroform (Camlab) and chloroform/isoamyl alcohol (BDH) at 24:1 (Sambrook, Fristsch & Maniatis, 1989). The EDTA was removed by centrifugal dialysis with Microcon-30 microconcentrators (Amicon) and the DNA was purified using guanidine thiocyanate/silica gel columns (Boom *et al.*, 1990; Wizard DNA clean up system, Promega). One extraction blank, consisting of all the reagents except the bone powder, was performed per batch of extractions. Two independent extractions were performed for each sample. In the cases of 7946, 8260 and STORT, the extractions were performed from two different bones, and from one bone and one tooth for the subadult 11702.

DNA amplification

The amplifications were carried out as described (Richards, Sykes & Hedges, 1995), except that reactions were performed in 25 µl of reaction mixture consisting of KCl buffer (Bioline) including 15 mM $MgCl_2$, and that 0·25 units of BioTaq polymerase (Bioline) were used. Two overlapping primer pairs, conL3/conH3-B and conLC/conH2-B were used to amplify most of the first hypervariable segment of the mitochondrial DNA control region. ConL3/conH3-B amplify the region between positions 16,037 and 16,257, and conLC/conH2-B amplify the region between positions 16,190 and 16,421 (Richards, Sykes & Hedges, 1995). One of the primers of each pair was biotinylated at the 5′ end. For the amplification of a 513 bp fragment, conL1 (TCA AAG CTT ACA CCA GTC TTG TAA ACC) was used with conH2-B.

Between three and five amplification blanks were performed with each batch of PCR reaction. Cycling parameters were as described in Richards, Sykes & Hedges (1995). A second-round amplification of 25 cycles was performed from PCR products purified by electrophoresis on a 3% Nusieve (Flowgen) agarose gel, with the same primers as in the first round. Primers Y1.1 and Y1.2 were used to amplify a 149 bp fragment of the Y chromosome repeat DYZ1 (Kogan, Doherty & Gitschier, 1987; Hummel & Hermann, 1991). The cycling parameters were as for the mitochondrial DNA except that the annealing temperature was 54°C.

Amplification of the amelogenin gene was performed as described in Sullivan *et al.* (1993). Single-stranded templates for sequencing were isolated using magnetic beads coated with streptavidin (Dynal). The sequencing was performed with the Sequenase 2.0 sequencing kit (Amersham). The excavators' sequences were

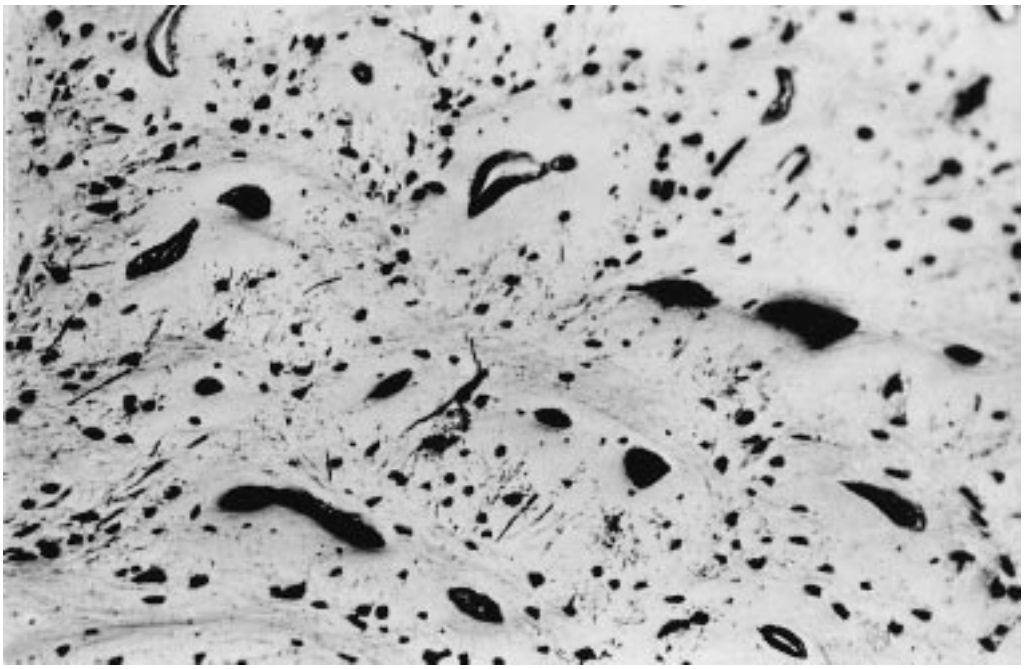

Figure 1. Histology of the infant bone W92-7946 (magnification 10 × 10).

determined to trace any possible contamination. The modern DNA control was added to the PCR tube independently from the samples.

Diversity and phylogenetic analysis

The calculation of the mean pairwise differences were computed with the MacPairwise 5·0 program (Macaulay & Micklem, 1995). Phylogenetic analysis was performed using full and reduced median networks (Bandelt *et al*., 1995).

Results

Bone diagenesis

The histology of the six infant bone fragments was examined. The structure of immature bone is somewhat different from adult bone, lacking the circular lamellae of the mature bone, and containing more cells (Vaughan, 1981). The histological appearance of one of the Wijnaldum infants' bones is shown in Figure 1. The preservation is excellent, without destructive foci, except for sample W92-STORT which shows some damage, and not dissimilar to modern infant bone structure. Therefore, applying Hedges bone histological index (Hedges, Millard & Pyke, 1995), our samples would merit a 4–5 score, and STORT a 3. The good preservation was supported by the N values (Table 1). The N content was determined for the six infant bones. The values were generally around 3% of N by weight, except W92-STORT which had a lower value of 1·98%. The value for modern adult bones is in the range of 4–5%.

Genetic sex determination

The genetic sex was determined using the DYZ1 system (Figure 2). The Y-chromosome region was clearly amplified for W92-8260, W93-9021, and W92-8008. These three individuals are thus identified as males. A faint band was present after the second-round amplification in the case of W92-STORT. The same result was obtained twice independently, which indicates that this individual was also male. W93-11702, W92-7946 and W92-7379 did not yield any product for the DYZ1 region after two rounds of amplification. This could indicate either that they are females or that they failed to amplify due to the degradation of the DNA. In view of the successful amplification mtDNA for these samples, and the amplification of Y-chromosome DNA from the less well-preserved STORT, it seems reasonable to regard these results as female. The amelogenin system, which relies on amplification from single nuclear copies, failed to give results.

Table 1. C:N ratios and the percentage of N in bone samples. A C:N ratio of around 3 is characteristic of bone. The percentage of N of modern bone is usually around 4%

Sample No.	C:N	% N	Histology index
W92-7946	3·53	3·53	4–5
W92-8260	3·54	3·10	4–5
W92-STORT	3·88	1·98	3
W92-7379	3·61	3·49	4–5
W93-9021	4·19	3·19	4–5
W92-8008	3·37	3·27	4–5

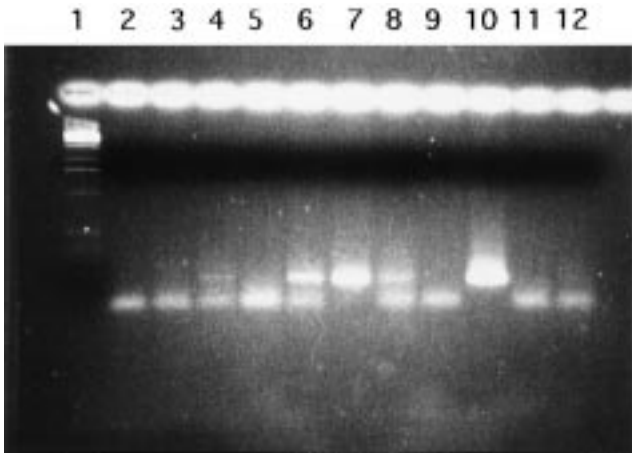

Figure 2. Agarose gel showing the second-round amplification products with the primers Y1.1/Y1.2 (DYZ1). Lane 1: 1 kb ladder; lane 2: W93-11702; lane 3: W92-7946; lane 4: W92-STORT; lane 5: W92-7379; lane 6: W92-8260; lane 7: W93-9021; lane 8: W92-8008; lane 9: extraction blank; lane 10: modern male DNA; lane 11: modern female DNA; lane 12: amplification blank.

Mitochondrial sequence data

All samples except 7946 and STORT were tested for amplification of a 513 bp fragment. None of them could be amplified. We have obtained complete sequences for the region 16,090–16,400 (Anderson *et al.*, 1981) for all individuals, from the two smaller fragments. The results are summarized in Table 2.

None of the ancient sequences matches the sequences of the excavators or of the geneticists who carried out the work in Oxford. They are also different from the modern DNA control which shows three transitions at positions 242, 270 and 356.

Our data were combined with modern population data to generate phylogenetic networks. Figure 3 shows our samples placed in a reduced median network constructed from the 82 haplotypes occurring at frequency two or more in Europe (Di Rienzo & Wilson 1991; Piercy *et al.*, 1993; Pult *et al.*, 1994; Richards *et al.*, 1996). Of our seven individuals, three (W93-11702,

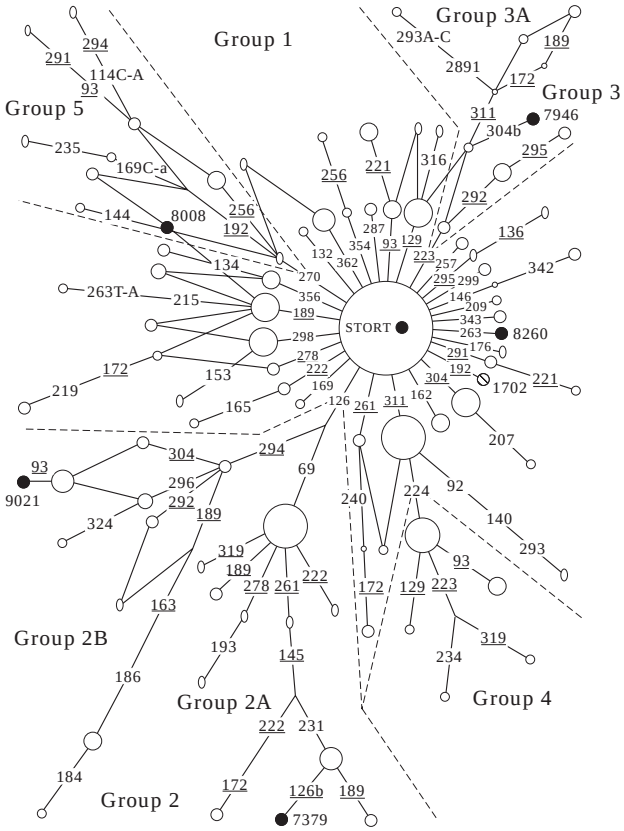

Figure 3. European skeleton network: a reduced median network constructed from the 82 haplotypes occurring at frequency two or more in Europe, using the data of Richards *et al.* (1996); Di Rienzo & Wilson (1991); Piercy *et al.* (1993) and Pult *et al.* (1994). The filled circles represent the Wijnaldum sequences. Circles represent sequence haplotypes for the mtDNA control region 16,090–16,365. The area of the circle is proportional to the frequency of the haplotype (except for the Wijnaldum sequences). The smallest circles are singly occurring haplotypes from a global database which fill nodes in the skeleton. Nodes remaining empty after this search are represented by black points. Lines represent mutational events between haplotypes and are labelled with the positions of transitional mutations using the numbering system of Anderson *et al.* (1981), less 16,000. Transversional mutations are labelled 't' and further specified. Dotted lines delineate the five lineage groups identified.

Table 2. Results of mitochondrial DNA analysis. All individuals were newborn except W93-11702. The table shows the number of PCR products that were sequenced from each extraction, the positions of the bases which could be read from the sequences, numbered as per Anderson et al. *(1981) less 16,000, and the transitional variants from the CRS. Note that position 391 is excluded from the network as it is outside the region sequenced for modern samples*

Sample No.	1st extraction	2nd extraction	Bases sequenced	Variants
W93-11702	2	1	90–400	192
W92-7946	1	1	90–400	129 223 391 304
W92-8260	2	1	90–400	263
W92-STORT	2	1	90–400	391
W92-7379	2	1	90–400	145 231 261
W93-9021	2	1	90–400	93 126 294 296 304
W92-8008	2	1	90–400	189 270
Excavator 1				265 (A-C transversion)
Excavator 2				189
Geneticist 1				CRS
Geneticist 2				93 231 256 270
Geneticist 3				224 311

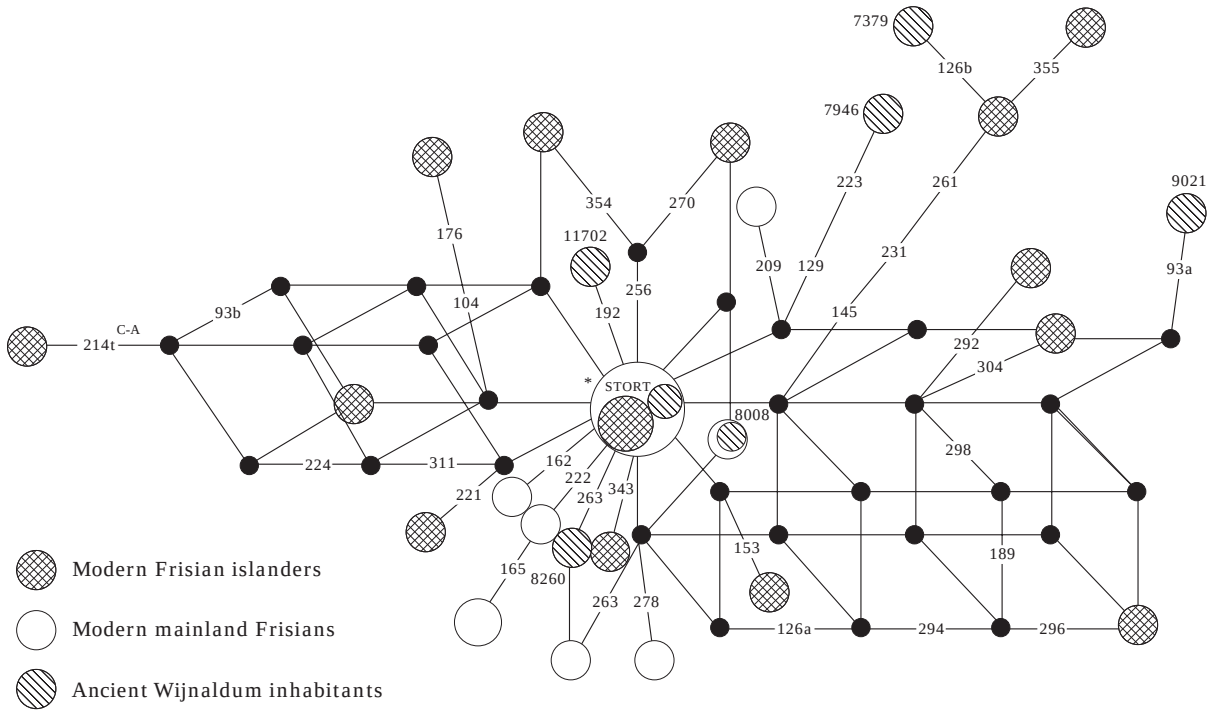

Figure 4. Median network showing the seven sequences from the terp and 28 modern Frisian speakers (16 North Frisian islanders, four German East Frisians, three German Saterlanders, and three Dutch West Frisians). Circles represent sequence haplotypes for the mtDNA control region 16,090–16,365. The area of the circle is proportional to the frequency of the haplotype; small dots represent hypothetical intermediate nodes. The network was reduced when incompatible haplotypes were separated by three or more events. Haplogroup 1B–5 are marked; remaining lineages fall into Group 1. The starred haplotype is the CRS. Reticulations in the network indicate regions of ambiguity in the branching structure.

W92-8260 and W92-STORT) appear to be members of Group 1, a cluster which we believe may have expanded throughout Europe during the Upper Palaeolithic, possibly in the immediate post-glacial. Sample 7379 falls into Group 2A, a cluster we believe to be associated with the Neolithic penetration from the Middle East, as it is relatively rare and homogenous in Europe but common and diverse in the Middle East. The sequence obtained here has never been found in modern Europeans but seems to fit into one branch of this cluster, characterized by transitions at positions 126, 145, 231 and 261. However, it lacks the variant at position 126. This position seems to have reverted. Sample 9021 falls into Group 2B, which we believe to be an Upper Palaeolithic founder group with its origins in the Middle East. Sample 7946 appears to be a member of Group 3, an apparently ancient cluster with its origin in the Middle East or even in Africa. Sample 8008 has a transition at positions 189 and 270, and is therefore a member of Group 5, an ancient European cluster whose origin we have not been able to trace. We therefore have found in the seven samples representatives of four of the five major lineage groups which we believe characterize European populations. This is one indication of a possibly elevated level of diversity amongst Frisians.

In Figure 4, we added our samples to a network of modern Frisian speakers from the North Frisian islands and the North German and Dutch mainland (Richards *et al*., 1995). The mean pairwise difference between the Wijnaldum individuals across the region 16,090–16,365 is $4{\cdot}19 \pm 0{\cdot}91$. This diversity is comparable to the Frisian islanders; $4{\cdot}48 \pm 0{\cdot}41$. By comparison, the diversity of mainland Frisian speakers is much lower, at $2{\cdot}42 \pm 0{\cdot}30$.

Discussion

The major problem when studying ancient human DNA is to assess the authenticity of the results. Contamination by modern human DNA can occur from the excavation site to the laboratory and is sometimes difficult to distinguish from an authentic result. However, several lines of evidence suggest that these results are authentic.

The sequences we obtained for the seven individuals all differ from each other. Moreover, they are different from those of all the people who handled the bones, either on the excavation site or in the laboratory. Also, we repeatedly failed to amplify single-copy genes or longer fragments of multicopy mitochondrial DNA, which seems to indicate that we were dealing with fragmented DNA and not with modern intact DNA. The absence of any amplified DNA in our extraction blanks or amplification blanks rules out the possibility

of carry-over contamination from former amplifications. DNA from each individual has been extracted twice independently, and sequenced twice from each of the extractions (except for 7946), always with the same results. It is thus highly unlikely that the variants could be artefactual. The sexing of the subadult is in agreement with studies based on morphological data.

The good preservation of the samples and their lack of contamination have been key factors for the success of this study. The histological preservation and the N content of the remains were very good. These qualities, especially the histology, have been shown to be correlated with the recovery of ancient DNA (Colson *et al.*, 1997). In this particular study, the individual showing the lowest value for the N percentage and the most damaged histological structure, W92-STORT, is the one for which the amplification was the least successful. This is especially true in the case of the sexing (Figure 1), but the same phenomenon was observed with mitochondrial DNA (data not shown). The Wijnaldum soil consists of an anoxic chalk with a high concentration of organic materials and phosphate (D. Gerrets, pers. comm.), which is an appropriate environment for bone tissue preservation thanks to the lack of oxygen (Millard, 1993). The presence of a high number of cells in infant bones (Vaughan, 1981) might also improve the chances of DNA recovery.

The importance of clean conditions during excavation and subsequent handling of the remains for the study of ancient DNA has to be emphasized. One should be especially very cautious while studying museum material, since contamination in this case is likely to be extensive. Moreover, old contaminant DNA could be somewhat degraded, mimicking ancient DNA and thus being impossible to recognize as such. The study of ancient human DNA, therefore, seems more promising in close collaboration with archaeologists on newly excavated material. Despite their very good state of preservation, we failed to amplify single-copy genes from any of the Wijnaldum individuals, indicating that results for such loci should be considered with great care.

An important contribution that the study of ancient DNA can provide in the case of pre-pubescent burials is the determination of sex, which is impossible to deduce from bone morphology. From the six infant skeletons studied, we were able to identify four males. The two other infants, as well as the subadult, were diagnosed as females. The genetic and morphological sex of the subadult were concordant. The diagnosis of genetic females is less reliable than males, because in the case of the DYZ1 it relies on an absence of amplification products, though concurrent amplification of mtDNA serves as an internal control for the presence of amplifiable DNA. Unfortunately, the amelogenin gene, which amplifies a fragment of different size from the two sex chromosomes, is a single-copy nuclear gene and thus is not as likely to be recovered from archaeological remains. This was the case in this study where, despite the good sample preservation, this method failed to give results.

The site at Wijnaldum is notable for the replacement of old Frisian by Anglo-Saxon material culture, and the change is sufficiently great to implicate a population replacement (Gerrets, 1995). Of the seven individuals, two are dated before the putative population replacement, four are dated after it, and one is of unknown date. This is too small a sample to address the question of such a replacement. Our sample is characterized by a very high diversity, having representatives of all the major lineage groups present in Europe, with the exception of Group 4. In this sense, they are more like extant Frisian speakers from the North Frisian islands, than mainland Frisian speakers (Bandelt *et al.*, 1995). This shared high level of diversity with modern Frisian speakers from the islands, when compared to modern mainland Frisian speakers, suggests a closer connection of the ancient Wijnaldum population to the modern Frisian islanders rather than to the modern populations of the Wijnaldum region.

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